Thrombospondin-1 Plays a Critical Role in the Induction of Hair Follicle Involution and Vascular Regression During the Catagen Phase

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Hair growth is associated with pronounced vascular-endothelial-growth-factor-induced perifollicular angiogenesis, whereas the catagen regression phase is characterized by apoptosis-driven blood vessel regression. The biologic relevance of endogenous inhibitors of angiogenesis in the control of hair cycling, however, has remained unknown. We studied the expression and biologic role of the angiogenesis inhibitor thrombospondin-1 (TSP-1) during the induced adult hair follicle-cycle in wild-type, TSP-1 deficient, and TSP-1 overexpressing transgenic mice. TSP-1 expression was absent from hair bulb and dermal papilla cells during early to mid-anagen but was highly upregulated throughout the catagen involution phase. In TSP-1 deficient mice, the follicle growth phase was significantly prolonged, associated with increased perifollicular vascularization and vascular proliferation. Conversely, hair follicle growth was delayed in K14/TSP-1 transgenic mice that expressed high levels of TSP-1 in outer root sheath keratinocytes, associated with reduced perifollicular vascularization. These effects were most probably mediated via its antiangiogenic effects because TSP-1 did not affect the growth of cultured murine vibrissae in the absence of a functional vascular system. These results identify a critical role of TSP-1 in the induction of anagen follicle involution, with potential implications for the therapeutic modulation of hair follicle growth. ***Key words: TSP-1/angiogenesis/VEGF. J Invest Dermatol 120:14–19, 2003***

The hair follicle undergoes a life-long cyclic transformation from the resting phase (telogen) to the growth phase (anagen), associated with rapid proliferation of follicular keratinocytes and with elongation and thickening of the hair shafts, followed by the regression phase (catagen) that leads to involution of hair follicles (Chase, 1954; Hardy, 1992). These changes involve rapid remodeling of both epithelial and dermal components and have led to the establishment of the murine hair follicle cycle as a prime model for studies of epithelial–mesenchymal interactions, leading to the identification of several important molecular mediators that control epithelial morphogenesis and growth (Paus and Cotsarelis, 1999). We have previously reported a pronounced increase in perifollicular vascularization during the anagen growth phase, accompanied by upregulation of vascular endothelial growth factor (VEGF) mRNA expression in follicular keratinocytes of the outer root sheath (Yano *et al*, 2001). Conversely, during the catagen follicular involution phase, a rapid regression of perifollicular blood vessels is associated with downregulation of follicular VEGF expression (Yano *et al*, 2001). Moreover, enhanced levels of VEGF in outer root sheath keratinocytes resulted in accelerated follicle growth and in increased thickness of hair follicles and hair shafts in K14/VEGF transgenic mice, whereas systemic inhibition of VEGF led to a reduced thickness of hair follicles (Yano *et al*, 2001).

During the catagen involution phase, hair follicles undergo a highly controlled process of involution that reflects a burst of programmed cell death (apoptosis) of follicular keratinocytes and of perifollicular endothelial cells (Paus and Cotsarelis, 1999; Mecklenburg *et al*, 2000; Yano *et al*, 2001). Our previous findings suggested that the regression of perifollicular blood vessels might represent a key factor that triggers the regression of hair follicles, resulting in reduced nutritional supply of proliferating hair matrix cells (Yano *et al*, 2001). Importantly, reduced vascularization of hair follicles has been suggested to play a critical role in disorders characterized by hair loss (Levy-Frankel, 1931; Beurey *et al*, 1971; Goldman *et al*, 1995), including androgenetic alopecia (male pattern baldness), which is associated with progressive shortening of successive anagen cycles and with miniaturization of genetically predisposed follicles (Cormia and Ernyey, 1961). Whereas the vascular expansion during anagen is probably due to upregulation of follicular VEGF expression, it has remained unknown whether endogenous inhibitors of angiogenesis might contribute to the vascular regression during catagen, and whether angiogenesis inhibition might represent a key event in the induction of hair follicle involution.

Thrombospondin-1 (TSP-1) is a 450 kDa matricellular protein that inhibits endothelial cell proliferation and migration *in vitro*

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Abbreviations: TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

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and angiogenesis associated with tumor growth and granulation tissue formation *in vivo* (Tolsma *et al*, 1993; Bleuel *et al*, 1999; Streit *et al*, 1999; 2000; Bornstein, 2001). TSP-1 is expressed in human and murine skin and contributes to the normal quiescence of the cutaneous vasculature (Detmar, 2000). To directly investigate the biologic role of TSP-1 in the control of hair follicle regression and vascular involution during catagen, we studied the induced adult hair follicle cycle in transgenic mice overexpressing TSP-1 in outer sheath follicular keratinocytes (Streit *et al*, 2000; Hawighorst *et al*, 2002; Yano *et al*, 2002) and in TSP-1 deficient mice (Lawler *et al*, 1998).

Here, we report that TSP-1 mRNA and protein expression are highly upregulated in the dermal papilla and in epithelial cells during the catagen and telogen phases, whereas TSP-1 expression is absent from the hair bulb during the anagen growth phase. Importantly, transgenic overexpression of TSP-1 in follicular keratinocytes resulted in retarded hair growth, associated with decreased perifollicular vascularization. Conversely, TSP-1 deficient mice showed a prolongation of the anagen growth phase with increased perifollicular vascularization, accompanied by increased proliferation of perifollicular endothelial cells and hair matrix cells. Together with the absence of direct effects of TSP-1 in vibrissa organ cultures, our results identify a major role of TSP-1-mediated angiogenesis inhibition in the control of hair growth and cycling.

METHODS

Induced adult hair follicle cycle The adult hair follicle cycle was induced in the back skin of 8-wk-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) by depilation as described previously (Paus *et al*, 1990), resulting in a synchronized induction of anagen follicle growth. Tissue samples were obtained at days 1, 3, 5, 8, 12, 15, 18, 19, 20, and 22 after depilation as described previously (three mice per time point), encompassing hair development from early anagen to telogen (Yano *et al*, 2001). Back skin was harvested parallel to the paravertebral line and the distinct phases of hair follicle development were determined as described earlier (Dry, 1926; Hardy, 1992). Skin samples were either snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin as described previously (Streit *et al*, 2000). All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Hair follicle cycle studies in TSP-1 transgenic mice and in TSP-1 deficient mice TSP-1 transgenic mice were established by using a keratin 14 (K14) promoter expression cassette to target human TSP-1 expression to basal epidermal keratinocytes and outer root sheath keratinocytes of hair follicles. The establishment and the phenotypic characterization of TSP-1 transgenic mice have been previously reported (Streit *et al*, 2000). The induced hair follicle cycle was studied in 8-wk-old TSP-1 transgenic mice ($n = 30$) and in age-matched wild-type littermates ($n = 30$ per group) as described above. Hair follicle cycling was also investigated in TSP-1 deficient mice (Lawler *et al*, 1998) and their wild-type controls ($n = 10$ per group).

Immunohistochemistry and computer-assisted morphometric vessel analysis Immunohistochemical stainings were performed on 7 μm frozen sections as described previously (Streit *et al*, 2000), using a monoclonal rat antimouse CD31 antibody (Pharmingen, San Diego, CA) and a secondary antirat IgG antibody labeled with AlexaFluor488 (Molecular Probes, Eugene, OR). Representative sections obtained from three mice for each time point were analyzed, using a Nikon E-600 microscope (Nikon, Melville, NY). Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) and morphometric analyses were performed using the IP LAB software (Scanalytics, Fairfax, VA) as described previously (Streit *et al*, 1999). Three different fields in each section were examined at 60 × magnification, and the average vessel size and the relative area occupied by blood vessels were determined within an area of 30 μm distance of individual hair follicles as described previously (Yano *et al*, 2001). The two-sided unpaired *t* test was used to analyze differences between the different genotypes. Endothelial cell proliferation was studied by intraperitoneal injection of mice with 5-bromodeoxyuridine (BrdU; 250 mg per kg of body weight) 2 h prior to sacrifice, followed by double immunofluorescence staining with anti-BrdU (Pharmingen) and anti-CD31 antibodies (Streit *et al*, 2000).

Measurement of hair follicle length Hematoxylin–eosin stains were performed on 7 μm frozen sections of tissues obtained from the different stages of the induced adolescent hair follicle cycle (three mice each per time point). Five representative sections for each sample were analyzed. The length of hair follicles was measured as the distance from the bottom of hair bulbs with clearly visible dermal papilla to the epidermis (50 hair follicles for each time point), using the IP LAB software. Statistical analysis was performed using the two-sided unpaired Student's *t* test.

***In situ* hybridization** *In situ* hybridization was performed on 5 μm paraffin sections as described previously (Streit *et al*, 2000). Sense and antisense RNA probes to human TSP-1 were transcribed from a pBluescript II KS + vector containing a 240 bp PCR fragment of the coding region of human TSP-1. This probe recognizes both mouse and human TSP-1 (Streit *et al*, 2000). Transcription reactions were carried out using the Riboprobe Gemini II kit (Promega Madison, WI) in the presence of (α-^{35}S) UTP. TSP-1 mRNA expression was quantified in sections obtained from three mice for each time point by counting individual photographic grains over follicular keratinocytes as described by Brown *et al* (1995), and samples were scored as negative (0–1 grains per cell; score 0), weakly positive (2–5 grains per cell; score 1), moderately positive (6–10 grains per cell; score 2), or strongly positive (>10 grains per cell; score 3). Data are shown as percentage of maximum score.

Vibrissa organ culture Whisker pads were isolated from 5-wk-old female C57/BL mice as described previously (Yano *et al*, 2001) and were shortly immersed in 70% ethanol in phosphate-buffered saline, followed by incubation in William's E medium containing 400 U per ml penicillin, 400 μg per ml streptomycin, and 1 μg per ml fungizone (Life Sciences Rockville, MD) for 10 min. Vibrissa follicles in the early or mid-anagen growth phase were isolated under a dissecting microscope, and the part of the hair shaft that extended over the epidermal surface was cut off. Follicles were then plated on nylon membranes (ICN, Irvine, CA) in serum-free William's E medium alone ($n = 8$), in medium containing 10 μg per ml human TSP-1 ($n = 7$), or in medium containing 10 ng per ml transforming growth factor β2 ($n = 8$; Sigma, St. Louis, MO). Media were replaced by fresh medium every 2 d, and follicles were incubated over a total period of 7 d. After 7 d, the length of the outgrowing hair shafts was determined by image analysis of digital pictures, using the IP LAB software. Data are expressed as means ± SD of *in vitro* hair growth; statistical analyses were performed using the two-sided unpaired Student's *t* test.

RESULTS

Upregulation of TSP-1 mRNA expression during the catagen follicle involution phase *In situ* hybridization studies revealed low levels of TSP-1 mRNA expression in follicular keratinocytes of the outer root sheath during the anagen growth

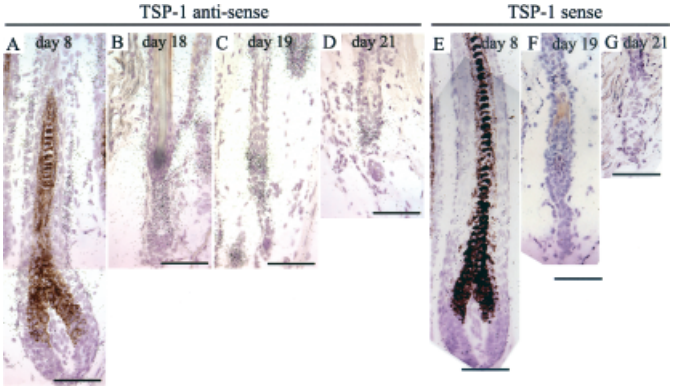

Figure 1. Upregulation of TSP-1 expression during catagen. *In situ* hybridization with TSP-1 antisense riboprobe reveals moderate TSP-1 mRNA expression in follicular keratinocytes of the outer root sheath (*arrows*), but not in the hair bulb, during the anagen follicle growth phase in wild-type mice (*A*). In contrast, TSP-1 expression was strongly upregulated in the dermal papilla and the epithelial strand throughout the catagen phase (days 18 and 19) (*B, C*) and was maintained during the telogen resting phase (*D*). Sense control hybridizations demonstrate low background signal (*E–G*). *Scale bars*: 50 μm.

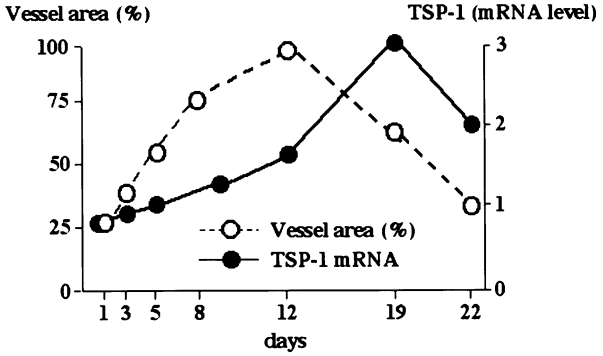

Figure 2. *In situ* hybridization revealed an inverse correlation of TSP-1 mRNA expression levels (*filled circles*) by dermal papilla cells and perifollicular angiogenesis. Relative vessel area (*open circles*) is expressed as a percentage of the maximum vessel area detected during late anagen (day 12; compare to **Fig 3*C***).

phase (**Fig 1*A***), whereas no signal was detected in the hair bulb or the dermal papilla. In contrast, TSP-1 expression was upregulated in dermal papilla cells and in the epithelial strand throughout the catagen involution phase (days 18 and 19) (**Fig 1*B*, *C***). In telogen follicles, strong TSP-1 mRNA expression was predominantly found in the lower part of the hair follicle (**Fig 1*D***). Numerous apoptotic follicular keratinocytes and several apoptotic perifollicular endothelial cells were found during the catagen involution phase (data not shown).

We next evaluated whether enhanced TSP-1 expression levels during the hair follicle cycle were inversely related to the extent of perifollicular vascularization. To this end, we performed a semiquantitative analysis of TSP-1 mRNA expression, as assessed by *in situ* hybridization during the distinct phases of the hair follicle cycle, and temporally related the TSP-1 expression levels with the quantitative analysis of perifollicular vascularization. Whereas TSP-1 expression levels were low during the vascular-rich anagen growth phase from day 1 to day 12, at day 19 TSP-1 expression levels raised sharply, associated with a decrease of perifollicular vascularization (**Fig 2**). These analyses confirmed an inverse relation between TSP-1 expression levels and the extent of perifollicular vascularization (**Fig 2**), suggesting a role of TSP-1-mediated angiogenesis inhibition during hair follicle involution.

Impaired hair development in late anagen in K14/TSP-1 transgenic mice To directly examine the *in vivo* effect of TSP-1 on hair growth and cycling, we studied the induced adult hair follicle cycle in transgenic mice with selective overexpression of TSP-1 in basal epidermal keratinocytes and outer root sheath keratinocytes and in wild-type littermates. No major differences in hair regrowth were observed between the two genotypes during the first 8 d after depilation. After 12 d, TSP-1 transgenic mice showed significantly impaired hair regrowth with few visible hair shafts, compared with clearly visible hair growth in wild-type littermates (**Fig 3*A*, *B***). No major macroscopic differences between the two groups were found after 15 d. By histologic analysis, no major differences in the length of hair follicles were observed until day 8 (mid-anagen). At day 12, however, we found a significant 37.8% decrease ($p < 0.001$) of hair follicle length in TSP-1 transgenic mice (**Fig 3*C*, *D*, *G***), compared with wild-type mice. No further elongation of hair follicles was detected in TSP-1 transgenic mice during the late anagen phase (**Fig 3*E*, *F*, *G***).

Decreased perifollicular vascularization in K14/TSP-1 transgenic mice To investigate whether the delay and reduction of hair follicle growth in TSP-1 transgenic mice was associated with a suppression of perifollicular vascularization, we performed a computer-assisted image analysis of tissue sections

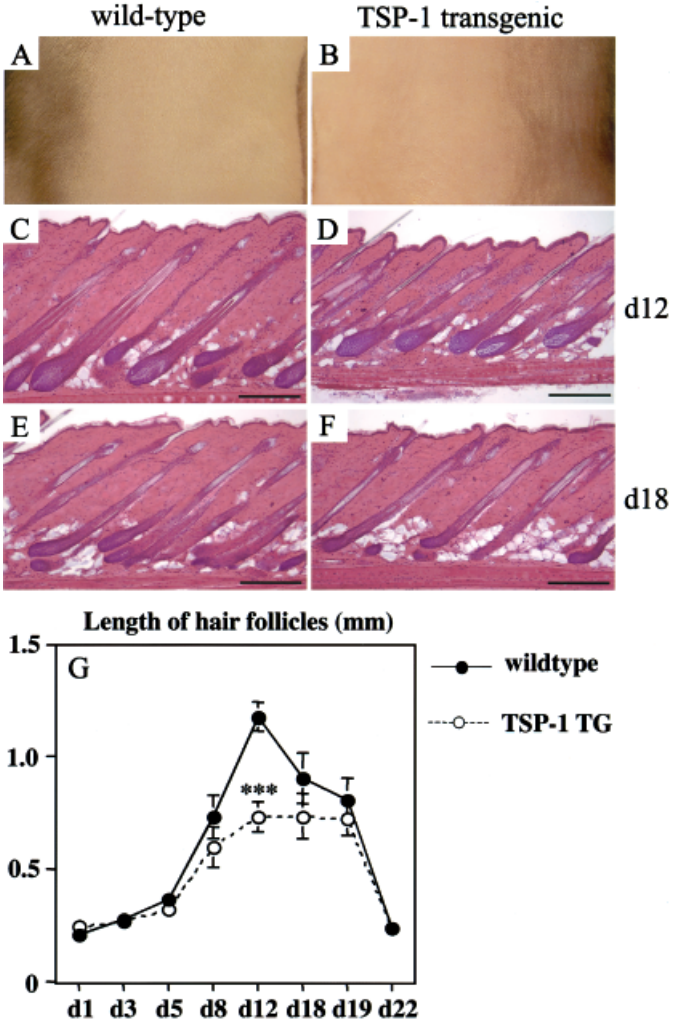

Figure 3. Impaired hair follicle growth in TSP-1 transgenic mice. At 12 d after depilation, hair regrowth was clearly visible in wild-type mice (*A*), but not in K14/TSP-1 transgenic mice (*B*). Histologic analysis reveals reduced length of anagen hair follicles in K14/TSP-1 transgenic mice (*D*) at this time point, compared with wild-type mice (*C*). At the onset of catagen involution (day 18), hair follicles were still shorter in TSP-1 transgenic mice (*F*) than in wild-type mice (*E*). (*C*)–(*F*), Hematoxylin–eosin stains; *bars*, 200 μm. (*G*) Morphometric analysis revealed a significant decrease of anagen hair follicle length in K14/TSP-1 transgenic mice at day 12 after depilation ($p < 0.0001$). Mean values ± SD; $n = 3$ per time point.

stained for the endothelial junction molecule CD31 (Dejana *et al*, 1995). At day 8 after depilation, we found a 48.2% decrease ($p < 0.001$) in vessel size (**Fig 4*C***) and a 20.2% decrease ($p < 0.05$) in the total area occupied by vessels (**Fig 4*D***) in K14/TSP-1 transgenic mice compared with wild-type littermates. These changes were even more pronounced after 12 d (late anagen **Fig 4*A*** and ***B***), with a 50.2% decrease ($p < 0.001$) in vessel size (**Fig 4*C***) and a 46.4% decrease ($p < 0.001$) in vessel area (**Fig 4*D***) in K14/TSP-1 transgenic mice. No major differences between the two genotypes were detected at later stages of the hair follicle cycle. Together, these results suggested that normal follicle development during the anagen growth phase is dependent on sufficient vascular support, and that suppression of follicular vascularization by TSP-1 suppressed hair follicle growth.

Delayed onset of catagen follicle involution in TSP-1 deficient mice The upregulation of TSP-1 expression during the late anagen phase and throughout catagen and telogen suggested that TSP-1 might play a major role in the induction of catagen follicle involution. Therefore, we next studied the hair

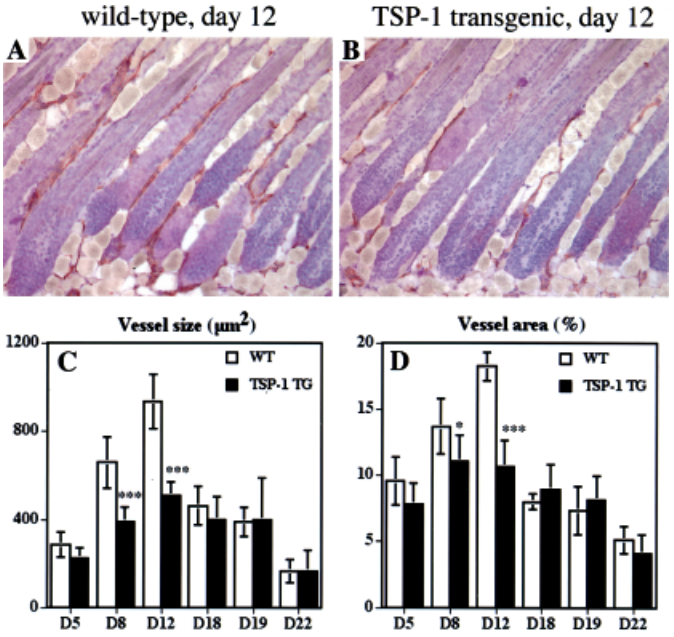

Figure 4. Diminished perifollicular angiogenesis in TSP-1 transgenic mice. CD31 immunostains (red) of wild-type (*A*) and TSP-1 transgenic (*B*) mice demonstrate diminished perifollicular vascularization of anagen follicles (day 12) in TSP-1 transgenic mice. *Scale bars*: 100 μm. Quantitative image analysis of CD31-stained vessels revealed a significantly decreased average vessel size ($p < 0.001$) at 8 and 12 d after depilation (*C*) in TSP-1 transgenic mice (*filled bars*) compared with wild-type mice (*open bars*) and a significant decrease of the relative area covered by vessels (*D*) at days 8 and 12 in K14/TSP-1 transgenic mice. $^{*}p < 0.05$; $^{***}p < 0.001$. Mean values ± SD; $n = 3$ per time point.

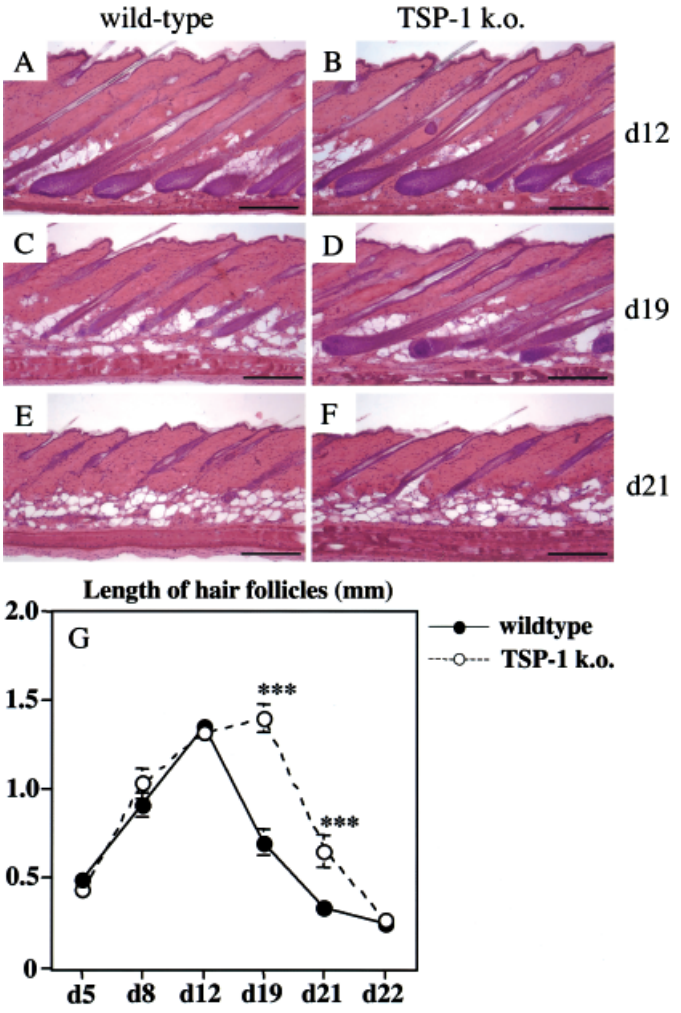

Figure 5. Prolonged anagen hair growth in TSP-1 deficient mice. No major differences in the length of hair follicles were detected in TSP-1 deficient mice and wild-type mice until day 12 of the induced hair cycle (*A*, *B*). At day 19, wild-type follicles had initiated catagen involution (*C*), whereas TSP-1 deficient follicles were still in the anagen growth phase (*D*). After 21 d, only catagen follicles were detected in both genotypes (*E*, *F*), although TSP-1 deficient follicle length was still increased over wild-type follicles (*G*). Morphometric analysis revealed a significant increase of hair follicle length in TSP-1 deficient mice at days 19 and 21 after depilation. $^{***}p < 0.001$. Mean values ± SD; $n = 3$ per time point. *Scale bar*: 100 μm.

follicle cycle in mice with targeted gene disruption of the TSP-1 gene. We found comparable growth of hair follicles in TSP-1 deficient and in wild-type mice during the anagen growth phase (**Fig 5*A*, *B***). The onset of catagen involution was significantly delayed in TSP-1 deficient mice, however. Whereas all follicles had initiated catagen involution at day 19 in wild-type mice, only anagen follicles with a clearly recognizable dermal papilla were detected in TSP-1 deficient mice at this time point (**Fig 5*C*, *D***). After 21 d, catagen involution was also detectable in TSP-1 deficient mice (**Fig 5*F***); however, the follicle length was still significantly increased ($p < 0.001$) compared with wild-type mice (**Fig 5*E*, *F***). After 22 d, all follicles in wild-type and in TSP-1 deficient mice were in the telogen resting phase.

Increased perifollicular vascularization in TSP-1 deficient mice Analysis of CD31-stained tissue sections revealed elongated and nlarged perifollicular blood vessels at day 19 in TSP-1 deficient mice, compared with small and fragmented vessels in wild-type mice. By quantitative image analysis of CD31-stained vessels, no major differences in vessel size and cutaneous area covered by blood vessels were observed until day 12 after depilation. We found a 110.3% increase ($p < 0.001$) in vessel size (**Fig 6*C***) and a 72.5% increase ($p < 0.05$) in total vessel area (**Fig 6*D***) during catagen at day 19, however, and a 60.3% increase ($p < 0.001$) in vessel size (**Fig 6*C***) and a 20.5% increase (n.s.) in vessel area (**Fig 6*D***) at day 21 in TSP-1 deficient mice, compared with wild-type littermates. Double immunofluorescence stains for CD31 and for the proliferation marker BrdU revealed persistent endothelial cell proliferation in TSP-1 deficient mice at day 19 (**Fig 6*B***), whereas no proliferating endothelial cells were detected in wild-type follicles that had already undergone catagen involution at this time point (**Fig 6*A***). The differences in vascularization between the two genotypes at day 19 were probably due to the prolongation of anagen growth in TSP-1 deficient mice, because no significant differences in the perifollicular vascularization were found in anagen phase follicles (**Fig 6*D***).

Absence of TSP-1 effects on hair growth and follicle size in vibrissa follicle cultures *in vitro* To investigate whether the observed inhibitory effects of TSP-1 on hair growth might have been mediated, in addition to its inhibitory effects on perifollicular angiogenesis, by direct effects of TSP-1 on hair follicle cells, we isolated mouse vibrissae and investigated hair growth in organ cultures *in vitro*, in the absence of a functioning vascular system. In organ culture, untreated mouse vibrissae showed an average hair shaft outgrowth of approximately 2.3 mm over a period of 7 d (**Fig 7**). Addition of 50 ng per ml transforming growth factor β to vibrissa cultures, used as a positive control, resulted in a significant ($p < 0.01$) inhibition of hair shaft growth, demonstrating the sensitivity of hair growth to inhibition in this experimental system (**Fig 7**). In contrast, addition of TSP-1 did not influence the hair growth rate in organ culture, suggesting that a functional perifollicular vascular

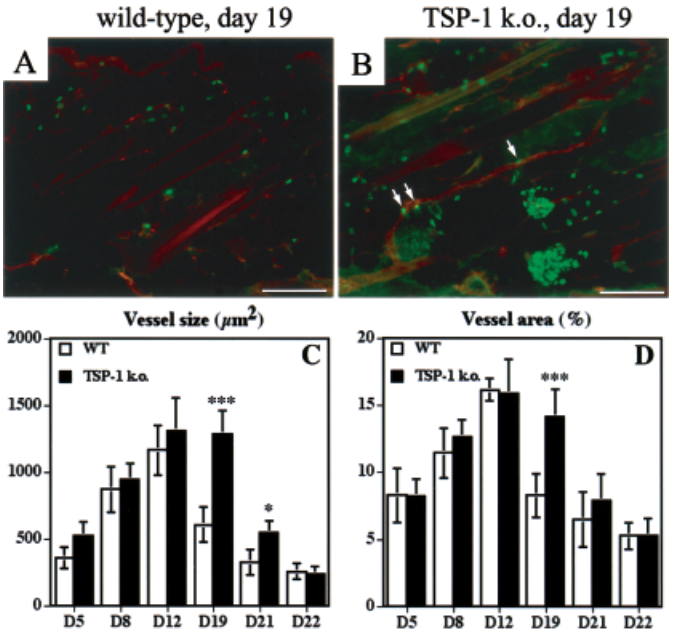

Figure 6. Increased perifollicular vascularization at day 19 in TSP-1 deficient mice. Double immunofluorescence stains for CD31 (red) and the proliferation marker BrdU (green) revealed proliferating endothelial cells (*arrows*) in the skin of TSP-1 deficient mice (*B*), but not in wild-type mice (*A*), at day 19 of the hair cycle. *Scale bar*: 100 μm. Increased perifollicular vascularization at day 19 in TSP-1 deficient mice. Computer-assisted morphometric analysis of CD31-stained sections revealed a significant increase ($p < 0.001$) in perifollicular vessel size (*C*) and relative vessel area (*D*) at day 19 in TSP-1 deficient mice (*filled bars*), compared with wild-type littermates (*open bars*). $^{*}p < 0.05$; $^{***}p < 0.001$. Mean values ± SD; $n = 3$ per time point.

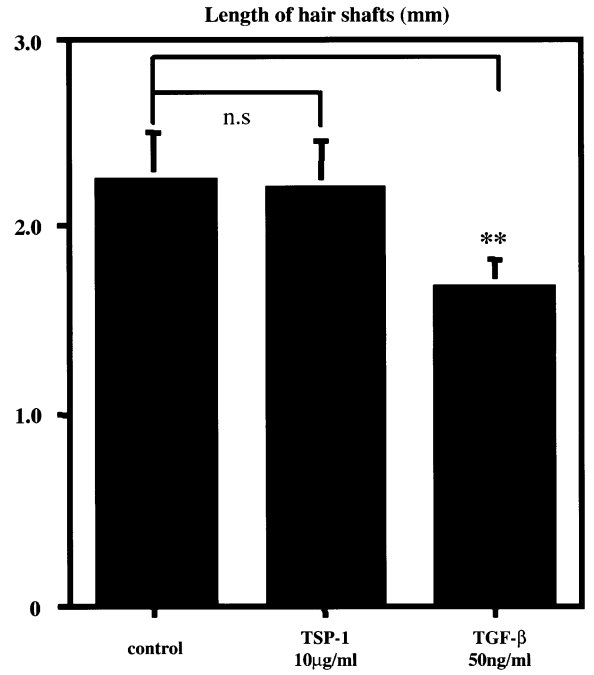

Figure 7. Absence of effects of TSP-1 treatment on the *in vitro* hair growth rate in mouse vibrissa organ cultures. Quantitative analysis of *in vitro* hair growth demonstrates significant inhibition of *in vitro* hair growth by 50 ng per ml transforming growth factor β (positive control) but lack of efficiency of TSP-1 (10 μg per ml) treatment. Data are expressed as mean ± SD. n.s., no significant differences; $^{**}p < 0.01$.

system is needed in order for TSP-1 to exert its inhibitory effects on hair growth.

DISCUSSION

Previously, we have reported that murine hair follicle growth is associated with pronounced perifollicular angiogenesis and VEGF expression by follicular keratinocytes of the outer root sheath (Yano *et al*, 2001). Moreover, increased VEGF levels in K14/VEGF transgenic mice resulted in increased hair shaft and follicle size, whereas systemic blockade of VEGF impaired hair follicle growth (Yano *et al*, 2001). The biologic role of endogenous inhibitors of angiogenesis for hair follicle growth and cycling has remained unknown, however. Based on our previous identification of TSP-1 as a major endogenous inhibitor of skin angiogenesis (Detmar, 2000; Streit *et al*, 2000; Hawighorst *et al*, 2002; Yano *et al*, 2002), we hypothesized that TSP-1 might also play an important role in the regulation of cyclic angiogenesis associated with hair follicle growth and involution. Semiquantitative *in situ* hybridization studies revealed that TSP-1 mRNA expression was relatively low during the anagen growth phase but was strongly upregulated in the dermal papilla and in follicular epithelial cells at the end of anagen and throughout the catagen involution phase and the telogen resting phase of the follicle. The inverse relation of TSP-1 mRNA expression levels with the levels of perifollicular angiogenesis suggests that TSP-1 represents a major antiangiogenic stimulus responsible for vascular involution during the catagen follicle involution phase.

To directly evaluate the biologic consequences of enhanced TSP-1 expression for hair growth, we analyzed the induced adolescent hair follicle cycle in our previously characterized transgenic mouse model for the selective overexpression of TSP-1 in the epidermis and outer root sheath follicular keratinocytes, using a K14 promoter to target transgene expression (Streit *et al*, 2000). We have previously reported decreased vascularization of granulation tissue during wound healing (Streit *et al*, 2000), reduced ultraviolet-B-induced cutaneous photodamage (Yano *et al*, 2002), and diminished epithelial carcinogenesis (Hawighorst *et al*, 2002) in these mice; however, hair growth had not been studied in detail. Our findings that targeted overexpression of TSP-1 in follicular keratinocytes resulted in delayed and diminished hair regrowth, associated with diminished perifollicular vascularization and increased levels of endothelial apoptosis, provide the first direct evidence that endogenous inhibitors of angiogenesis negatively regulate hair growth. Because TSP-1 treatment did not affect hair growth in organ cultures of mouse vibrissae, in the absence of a functional vascular system, our results suggest that the effects of TSP-1 are predominantly mediated through inhibition of perifollicular angiogenesis rather than by direct effects on hair follicles. These findings are in agreement with our previous results that systemic blockade of the angiogenesis factor VEGF with neutralizing antibody caused impaired anagen hair growth and decreased angiogenesis (Yano *et al*, 2001). We cannot exclude the possibility that TSP-1, at doses higher than those tested (10 μg per ml), might affect hair growth in vibrissa cultures, however, and that hair follicles might react differently to TSP-1, compared with vibrissa follicles. Moreover, because the localization of TSP-1 receptors in keratinocytes of the regressing hair follicles is unknown, potential direct effects of TSP-1 on intrafollicular cells cannot be completely ruled out.

The important role of TSP-1 in the control of hair follicle cycling was confirmed in additional studies on TSP-1 deficient mice that showed delayed induction of the catagen involution phase associated with maintained perifollicular angiogenesis, indicating that TSP-1 is a major part of the molecular program that initiates catagen follicle involution. In contrast to other mediators such as transforming growth factor β that have been previously

shown to be involved in the catagen hair involution (Foitzik *et al*, 2000), however, TSP-1 does not appear to directly inhibit hair follicle growth. Although the duration of the anagen growth phase was prolonged in TSP-1 deficient mice, we found that hair follicles had reached the telogen phase in both genotypes at day 22, suggesting that additional signals are involved in the control of the duration of the complete hair follicle cycle. Our recent studies suggest that the related angiogenesis inhibitor TSP-2 is also upregulated during catagen (unpublished results); therefore, TSP-2 expression might have partially compensated for the absence of TSP-1 in our studies. Future investigations in mice deficient for both TSP-1 and TSP-2 should provide valuable insights into the relative contribution of the two molecules to hair follicle cycle control.

Decreased VEGF mRNA expression was detected in human hair follicles in androgenetic alopecia (Goldman *et al*, 1995), and several previous studies have reported diminished follicular vascularization in conditions associated with impaired hair growth in humans (Goldman *et al*, 1995). Our results warrant further studies to investigate whether endogenous angiogenesis inhibitors such as TSP-1 might also be involved in human diseases associated with hair loss. In summary, our results establish an important functional role of TSP-1 in hair biology, and they provide additional support for our previous proposal that normal hair growth and cycling are dependent on sufficient perifollicular angiogenesis (Yano *et al*, 2001). Because TSP-1 was found to play a critical role in the induction of catagen follicle regression through its inhibitory effects on angiogenesis, these findings identify TSP-1 as a potential new target for therapies aimed at modulating hair follicle growth.

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